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13. ABSTRACT ***(Maximum 200 Words)***

Amplification and overexpression of the HER-2/neu gene were found in 20-30% of patients with breast cancer, and is an indicator for poor prognosis of the disease. To target the breast cancer cells-overexpressing HER-2/neu mRNA, a novel approach was developed. We combine the antisense principle and the biochemical property of a translation regulator, an iron-responsive element (IRE) to preferentially express therapeutic gene in HER-2/neu-overexpressing breast cancer cells. Briefly, IRE, when placed 5' to a gene, functions as a negative translation regulator in that IRE interacts with iron-regulatory proteins (IRPs) and this protein-RNA complex blocks translation. One way to alleviate this translation inhibition is to prevent the IRE/IRP interaction by disrupting the IRE stem-loop structure via a sense-antisense hybrid. Thus, we designed a HER-2/neu antisense IRE (AS-IRE) that possesses the IRE consensus sequence and functions as a translation inhibitor. When placed 5' to a reporter gene, AS-IRE could direct the reporter gene expression in breast cancer cells that overexpress HER-2/neu mRNA, because the AS-IRE-mediated translation inhibition can be overcome by the overexpression of HER-2/neu mRNA. In this project, we attempt to demonstrate the utility of this novel approach *in vitro* and *in vivo* by specifically directing gene expression in HER-2/neu-overexpressing breast cancer cells. During the first year of funding, we have finished the proposed goal stated in **Task 1** by identifying the "optimal" HER-2/neu antisense IRE, i.e., AS-IRE-4. We demonstrated that AS-IRE-4 could interact with IRP-1 and behave as a translational inhibitor when placed in the 5'UTR of a gene. More importantly, we showed that AS-IRE-4 could preferentially direct gene expression in the HER-2/neu-overexpressing cancer cells. These results represent a significant milestone that has paved the way for the construction of AS-IRE-4-directed therapeutic gene expression vector (**Task 2**). In addition to using DT-A, we recently cloned YCD gene and showed that YCD is functional in cell killing in the presence of 5-FC. Based on the encouraging results obtained from the reporter assay, we will next test if the AS-IRE-4-directed DT-A and YCD expression vectors could preferentially kill HER-2/neu-overexpressing cancer cells. The identification of AS-IRE-4 also facilitates our progress toward the final goal of this project: to test the therapeutic efficacy of the AS-IRE-4-directed DT-A and/or YCD expression vector in a pre-clinical gene therapy model (**Task 3**). We will use adenoviral vector and liposome as carriers to deliver the AS-IRE-4-directed therapeutic gene expression vector into the tumor-bearing mice. Treatment efficacy will be measured in terms of tumor size, survival, and metastatic lesions.

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Breast Cancer, HER-2/neu overexpression, iron-responsive element, pre-clinical gene therapy

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INTRODUCTION

The overexpression of HER-2/neu proto-oncogene has been found in a variety of human cancers. In particular, amplification and overexpression of the HER-2/neu gene were found in 20-30% of patients with breast cancer. These patients had decreased survival and increased relapse rates. Therefore, HER-2/neu overexpression has been used as a poor prognostic indicator for patients suffering from this disease. To develop an expression system that targets the breast cancer cells overexpressing HER-2/neu mRNA, a novel approach is described that combines the antisense principle and the biochemical property of a translation regulator, an iron-responsive element (IRE). An example of a typical IRE of human transferrin receptor mRNA is depicted in Fig. 1. IRE, when placed 5' to a gene, functions as a negative translation regulator in that IRE interacts with iron-regulatory proteins (IRPs) and this protein-RNA complex blocks translation (1). One way to alleviate this translation inhibition is to prevent the IRE/IRP interaction by disrupting the IRE stem-loop structure via a sense-antisense hybrid. Thus, a HER-2/neu antisense IRE (AS-IRE) possessing the IRE consensus sequence and functioning as a translation inhibitor was generated. When placed 5' to a reporter gene, AS-IRE could direct the reporter gene expression in breast cancer cells that overexpress HER-2/neu mRNA, since the IRE-mediated translation inhibition can be overcome by the overexpression of HER-2/neu mRNA. In this project, we attempt to establish the utility of this novel approach to targeting HER-2/neu-overexpressing breast cancer cells. Our goals are: (1) to obtain an optimal AS-IRE that directs the maximum expression of the reporter gene in HER-2/neu overexpressing breast cancer cells; (2) to demonstrate a preferential killing of HER-2/neu overexpressing breast cancer cells by using the optimal HER-2/neu antisense IRE to direct the expression of a toxin gene encoding diphtheria toxin A-chain (DT-A); and (3) to test the therapeutic effect of the AS-IRE-mediated DT-A expression vector *in vivo* by treating the mice that bear tumors with or without the overexpression of HER-2/neu gene.

BODY

(Figures are attached in the Appendices)

Task 1: To obtain an optimal HER-2/neu antisense IRE.

During the first year of funding, we have generated five AS-IREs constructs instead of three I originally proposed. The targeted regions of HER-2/neu mRNA are indicated in Fig. 2A. Fig. 2B shows the sequences of these AS-IREs, i.e., AS-IRE-1 ~ -5, and the positive control IRE, cIRE (Fig. 1). To test if AS-IREs are able to bind to the IRE binding protein, IRP-1, we performed a binding assay in which the radioactive-labeled *in vitro* synthesized AS-IREs were incubated with the recombinant IRP-1 protein (2, 3). The IRE/IRP-1 complex was subsequently analyzed in a gel-shift assay. As shown in Fig. 3, all the AS-IREs are capable of binding to IRP-1, suggesting that these AS-IREs maintain the integrity of both the sequence and stem-loop structure that are necessary for IRE/IRP-1 interaction *in vitro* (4). In particular, AS-IRE-4 exhibits the highest IRP-1 binding ability among the AS-IREs, including cIRE. On the other hand, AS-IRE-1 has the lowest IRP-1 binding activity.

To functionally demonstrate a AS-IRE/IRP interaction-mediated translational inhibition, we placed the AS-IREs in 5'UTR of a luciferase reporter gene to generate AS-IRE-Luc-1 ~ -5 driven by T7 promoter. We tested the *in vitro* translational inhibitory function of AS-IREs by using a transcription and translation coupling system (TNT) in wheat germ extract (WGE) that does not naturally contain IRP (5). A functional IRE should allow translation of luciferase mRNA in WGE but not in the presence of exogenous IRP. As shown in Fig. 4, in the absence of IRP, AS-IRE-Luc-4 has a 7-fold higher lucerase activity than that of CMV-Luc. It is possible that AS-IRE-4 may somehow enhance translation efficiency. The other AS-IREs either have no effect, e.g., AS-IRE-1, or have inhibitory effect, e.g., AS-IRE-2, -3, and -5, on translation efficiency. In the presence of IRP, like the positive control, cIRE-Luc, a significant translational inhibition was observed in AS-IRE-1, -4, and -5 when IRP is present. AS-IRE-2 and -3, however, do not exhibit significant translational inhibition by IRP. This *in vitro* data suggests that AS-IRE-1, -4, and -5 behave as a functional IRE that binds IRP and inhibits translation when placed in the 5'UTR of a mRNA. Among the AS-IREs, AS-IRE-4 is most sensitive to IRP-mediated translational inhibition, i.e., more than 30-fold reduction.

To identify the best AS-IRE that could direct luciferase expression in a HER-2/neu overexpression-specific manner *in vivo*, we transfected the AS-IRE-Luc plasmids into two breast cancer cell lines MCF-7 and MCF/HER2-18 (that is a stable transfectant overexpressing HER-2/neu gene) (6). These two cell lines contain a comparable level of IRP between each other as determined by a gel-shift assay (Fig. 5). We show in Fig. 6A that AS-IRE-Luc-4 has a significantly higher luciferase activity in MCF/HER2-18 than that in MCF-7, while the other AS-IRE-Lucs do not. This result suggests that AS-IRE-4 may be the best AS-IRE that could preferentially direct gene expression in HER-2/neu overexpressing breast cancer cells. This observation was confirmed when we transfected the AS-IRE-Luc plasmids into another HER-2/neu high- and low-expression system, i.e.,

two human ovarian cancer cell lines (7), 2774 (HER-2/neu low expressor) and SKOV3-ip1 (HER-2/neu high expressor). Again, AS-IRE-Luc-4 is the best among the AS-IRE-lucs in terms of the ability to direct luciferase expression in SKOV3-ip1 versus that in 2774 (Fig. 6B). Taken together, AS-IRE-4 appears to be the best AS-IRE that could preferentially direct gene expression in a HER-2/neu overexpression-specific manner as determined by our assay system.

We have attempted to modify the lower-stem of the AS-IRE structures, hoping that we could achieve a better targeting efficiency and HER-2/neu overexpression specificity. One way to accomplish that, we thought, was to increase the length of the lower stem, and thus generating longer sense/antisense hybrids. We chose AS-IRE-3 and -5 for such modification because both of them responded poorly to IRP *in vitro* (Fig. 4) and were insensitive to HER-2/neu overexpression *in vivo* (Fig. 6). We engineered the lower stems of AS-IRE-3 and -5 in a way that they resemble the lower stem of a classical ferritin IRE in which an additional CU-bulge is present in the lower stem (8). The modified structures are shown in Fig. 7A and 7B, in that the sense/antisense hybrid would result in 29 bp (designated as AS-IRE-3-1 and AS-IRE-5-1) and 36 bp (designated as AS-IRE-3-2 and AS-IRE-5-2). We also made AS-IRE-3-3 and AS-IRE-5-3 that would generate 70 bp sense/antisense hybrid including a 5' flanking sequence (not shown). These modified AS-IREs, when placed in 5'UTR of a luciferase gene, did not however improve the HER-2/neu overexpression specificity when they were tranfected into either MCF-7 and MCF/HER2-18 system (Fig. 8A) or 2774 and SKOV3-ip1 system (Fig. 8B). In fact, when transfected into another HER-2/neu low- (Rat1-pcDNA3) and high- (Rat1-HER2m) expression system, the longer the sense/antisense hybrid seems to have a more adverse effect on translation without apparent HER-2/neu overexpression specificity (Fig. 9). It is possible that a long sense/antisense hybrid might trigger a double-strand RNA-specific ribonuclease activity within the cell. Thus, a critical length of sense/antisense hybrid may exist to avoid such nuclease activity, and, more importantly, remains competent for translation. Therefore, instead of increasing the length of sense/antisense hybrid, we are currently modifying the AS-IRE structures so that we might identify a minimum length of sense/antisense hybrid that not only could abolish IRP/IRE complex, but maintain the specificity for HER-2/neu overexpression.

Task 2: To obtain an optimal HER-2/neu antisense IRE-regulated toxin gene (DT-A).

With the identification of the optimal AS-IRE, i.e., AS-IRE-4, we are in a position to generate a therapeutic gene expression vector directed by AS-IRE-4. Thus, we will place AS-IRE-4 in the 5'UTR of DT-A gene to generate AS-IRE-DT-A. However, given the relatively high background expression of AS-IRE-Luc-4 *in vivo* as compared to that of CMV-luc (Fig. 6A and 6B), it is likely that the expression of a potent toxin such as DT-A might kill both the HER-2/neu high- and low-expressing cells. To circumvent this potential problem, we employed an enzyme/pro-drug approach using yeast cytosine deaminase (YCD) and 5-fluorocytosine (5-FC) (9). YCD can efficiently convert 5-FC into the toxic metabolite 5-fluorouracil (5-FU). YCD/5-FC strategy has been used in

several cancer gene therapy systems (9, 10, 11). We have cloned YCD from yeast genomic DNA by PCR. To test the functionality of YCD, we placed YCD gene under the control of CMV and human telomerase (hTERT) (12) promoters, i.e., CMV-YCD and hTERT-YCD, respectively. We then transiently transfected these plasmids into HCT-116 cells, a lung cancer cell line. Using YCD-specific primers, RT-PCR was performed to detect the expression of YCD. As shown in Fig. 10, YCD expression (a 477 bp band) could be readily detected in both CMV-YCD- and hTERT-YCD-transfected cells, but not in the mock transfected control cells. GAPDH (a 190 bp band) serves as a internal control that shows a comparable level of total RNA used in the reactions. To demonstrate the function of YCD, we transiently transfected CMV-YCD and hTERT-YCD into HCT-116 cells with (0 mM) or without 5-FC (5 mM). Forty-eight hours after transfection, MTT assay was used to measure the cell viability. In the presence of 5-FC, cell killing was observed in the YCD-transfected cells, i.e., 15% for hTERT-YCD and 23% for CMV-YCD (Fig. 11A). We will systemically study the time course as well as the dose effect of YCD/5-FC approach to obtain the optimal assay condition for the subsequent experiments in our system. To confirm that our YCD clone is functional, we generated a CMV-YCD stable MCF-7 cell line (MCF-7/CMV-YCD). We grew MCF-7/CMV-YCD in the presence of 5-FC (0, 1, and 5 mM) for 24 hours, followed by MTT assay. As shown in Fig. 11B, a dose-dependent cell killing of MCF-7/CMV-YCD was observed. Taken together, our results indicate that we have obtained a functional YCD. We will subclone AS-IRE-4 into the 5'UTR of YCD gene to generate AS-IRE-YCD. We will then transfect AS-IRE-YCD into HER-2/neu high- and low-expressing cells and test if AS-IRE-YCD could preferentially kill HER-2/neu-overexpressing cells in the presence of 5-FC.

Task 3: To demonstrate the therapeutic effect of the HER-2/neu antisense IRE-mediated gene expression.

Once a successful demonstration of preferential killing in HER-2/neu-overexpressing cells is accomplished (see **Task 2**), we will test the therapeutic efficacy of AS-IRE-YCD/5-FC (or AS-IRE-DT-A) gene therapy treatment in a breast cancer xenograft model. Two gene delivery systems will be employed: liposome and adenoviral vector. AS-IRE-YCD/liposome complex or adenovirus expressing AS-IRE-YCD will be delivered into the tumor-bearing mice. Following 5-FC administration, we will measure the tumor size, survival, and metastatic lesions to determine the therapeutic efficacy of the AS-IRE-mediated gene therapy.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of HER-2/neu antisense IREs (AS-IREs).
- Demonstration of AS-IREs interacting with IRP-1 and the resulting translational inhibition *in vitro*.
- Identification of AS-IRE-4 being the best candidate for the optimal HER-2/neu antisense IRE.
- Demonstration of a HER-2/neu overexpression-specific expression directed by AS-IRE-4 *in vivo*.
- Cloning of YCD and demonstration of its *in vivo* killing in the presence of 5-FC.

REPORTABLE OUTCOMES

- Cloning of YCD
- Generation of a MCF-7 stable cell line expressing YCD, MCF-7/CMV-YCD.

CONCLUSIONS

During the first year of funding, we have finished the proposed goal stated in **Task 1** by identifying the "optimal" HER-2/neu antisense IRE, i.e., AS-IRE-4. We demonstrated that AS-IRE-4 could interact with IRP-1 and behave as a translational inhibitor when placed in the 5'UTR of a gene. More importantly, we showed that AS-IRE-4 could preferentially direct gene expression in the HER-2/neu-overexpressing cancer cells. These results represent a significant milestone that has paved the way for the construction of AS-IRE-4-directed therapeutic gene expression vector (**Task 2**). In addition to using DT-A, we recently cloned YCD gene and showed it is functional in cell killing in the presence of 5-FC. We will test if the AS-IRE-4-directed DT-A and YCD expression vectors could preferentially kill HER-2/neu-overexpressing cancer cells. The identification of AS-IRE-4 also facilitates our progress toward the final goal of this project: to test the therapeutic efficacy of the AS-IRE-4-directed DT-A and/or YCD expression vector in a pre-clinical gene therapy model (**Task 3**). We will use adenoviral vector and liposome as carriers to deliver the AS-IRE-4-directed therapeutic gene expression vector into the tumor-bearing mice. Treatment efficacy will be measured in terms of tumor size, survival, and metastatic lesions. Although AS-IRE-4 is our "optimal" AS-IRE at present, we will continue to modify AS-IRE-4, perhaps other AS-IREs, it is possible that we may identify even better AS-IREs that possess targeting efficiency and HER-2/neu overexpression specificity than AS-IRE-4.

REFERENCES

1. R. D. Klausner, Rouault, T. A., and Harford, J. B., *Cell* 72, 19-28 (1993).

2. E. A. Leibold, and Munro, H. N., *Proc. Natl. Acad. Sci. USA* 85, 2171-2175 (1988).

3. T. A. Rouault, Tang, C. K., Kaptain, S., Burgess, W. H., Haile, D. J., Samaniego, F., McBride, O. W., Harford, J. B., and Klausner, R. D., *Proc. Natl. Acad. Sci. USA* 87, 7958-7962 (1990).

4. B. R. Henderson, E. Menotti, C. Bonnard, L. C. Kuhn, *J Biol Chem* 269, 17481-9 (1994).

5. H. Hirling, et al., *Nucleic Acids Res* 20, 33-9 (1992).

6. C. C. Benz, Scott, G. K., Sarup, J. C., Johnson, R. M., Tripathy, D., Coronado, E., Shepard, H. M., and Osborne, C. K., *Breast Cancer Res. Treat.* 24, 85-95 (1992).

7. N. T. Ueno, et al., *Clin Cancer Res* 6, 250-9 (2000).

8. S. R. Jaffrey, Haile, D. J., Klausner, R. D., and Harford, J. B., *Nucleic Acids Res.* 21, 4627-4631 (1993).

9. D. A. Hamstra, D. J. Rice, S. Fahmy, B. D. Ross, A. Rehemtulla, *Hum Gene Ther* 10, 1993-2003 (1999).

10. L. D. Stegman, et al., *Proc Natl Acad Sci U S A* 96, 9821-6 (1999).

11. E. Kievit, et al., *Cancer Res* 59, 1417-21 (1999).

12. S. Kyo, et al., *Nucleic Acids Res* 28, 669-77 (2000).

APPENDICES

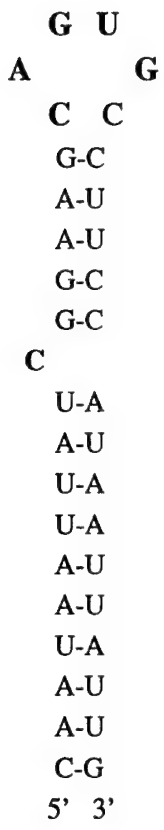
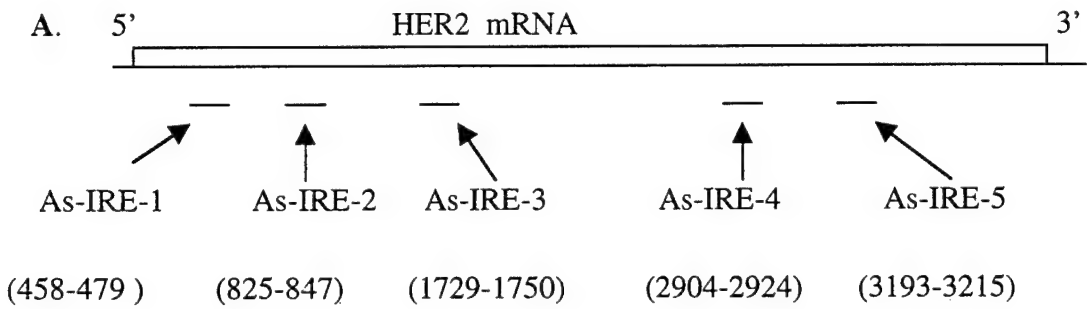


Fig.1 Structure of IRE(iron-responsive element) in the 3'UTR of human transferrin receptor (TfR) mRNA.

A.



B.

HindIII————————NotI (5'—3' after annealing)

(1) AS-IRE-1
5' AGCTTCAATCCGCAGCCTCTG**CAGTG**CCAGAGCTGCGGATTGGC 3'
(2) HER-2/neu -1
3' AGTTAGGCGTCGGAGACGTCACGGTCTCGACGCCTAACCGCCGG 5'

(3) AS-IRE-2
5' AGCTTCAGCCACCGGCACAGA**CAGTG**CTCTGTCCGGTGGCTGGC 3'
(4) HER-2/neu -2
3' AGTCGGTGGCCGTGTCTGTCACGAGACAGGCCACCGACCGCCGG 5'

(5) AS-IRE -3
5' AGCTTTGGGCCCTGACCCCAG**CAGTG**CCTGGGTCAGGGCCCAGC 3'
(6) HER/2neu -3
3' AACCCGGGACTGGGGTCGTCACGGACCCAGTCCCGGGTCGCCGG 5'

(7) AS-IRE -4
5' AGCTTGTCATCAGCTCCCACA**CAGTG**CTGTGGAGCTGATGACGC 3'
(8) HER2/neu -4
3' ACAGTAGTCGAGGGTGTGTCACGACACCTCGACTACTGCGCCGG 5'

(9) AS-IRE -5
5' AGCTTTGTCATCGTCCTCCAG**CAGTG**ACTGGAGACGATGACAGC 3'
(10) HER2/neu –5
3' AACAGTAGCAGGAGGTCGTCACTGACCTCTGCTACTGTCGCCGG 5'

(11) cIRE
5' AGCTTCAATAATTATCGGAAG**CAGTG**CCTTCCATAATTATTGGC 3'
(12) AS-cIRE
3' AGTTATTAATAGCCTTCGTCACGGAAGGTATTAATAACCGCCGG 5'

Fig.2 A. Targeting sites of five AS-IREs in human HER2/neu mRNA.
B. The DNA seguences of AS-IREs that have been artificially synthesized.

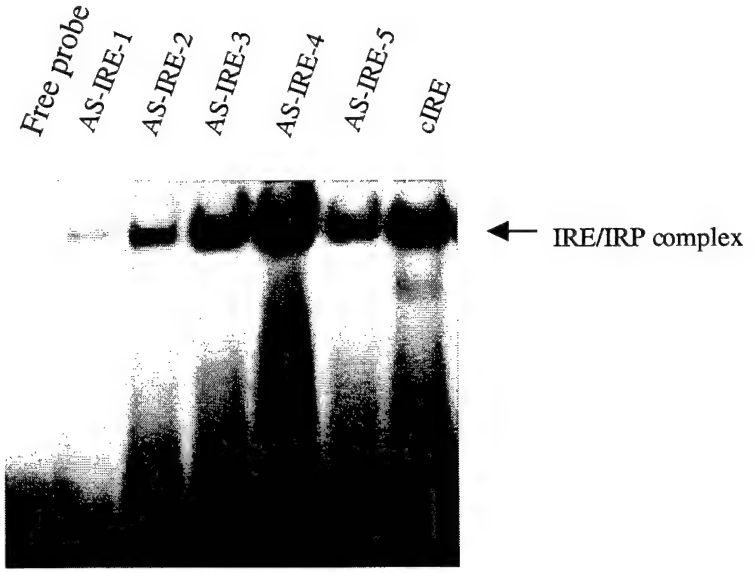


Fig.3 The result of RNA retardation assay. Each AS-IRE RNA probe was synthesized by T7 polymerase-drived in vitro transcription.

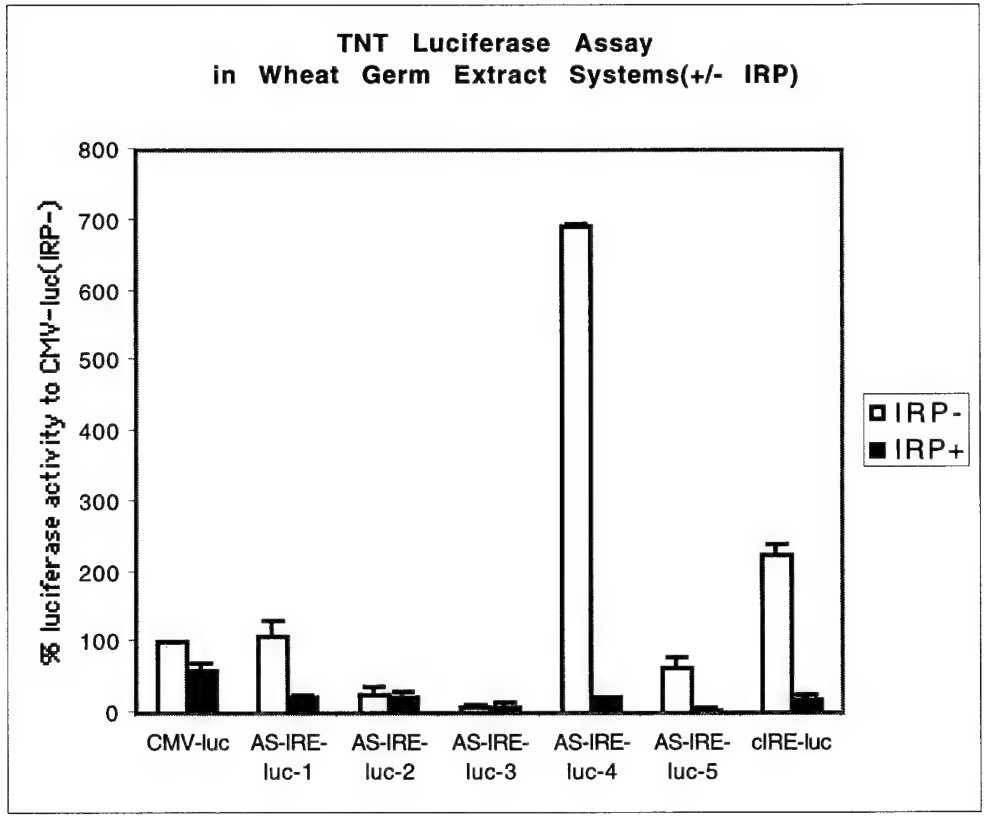


Fig.4 The result of luciferase assay in TNT wheat germ extract system (+/-IRP)

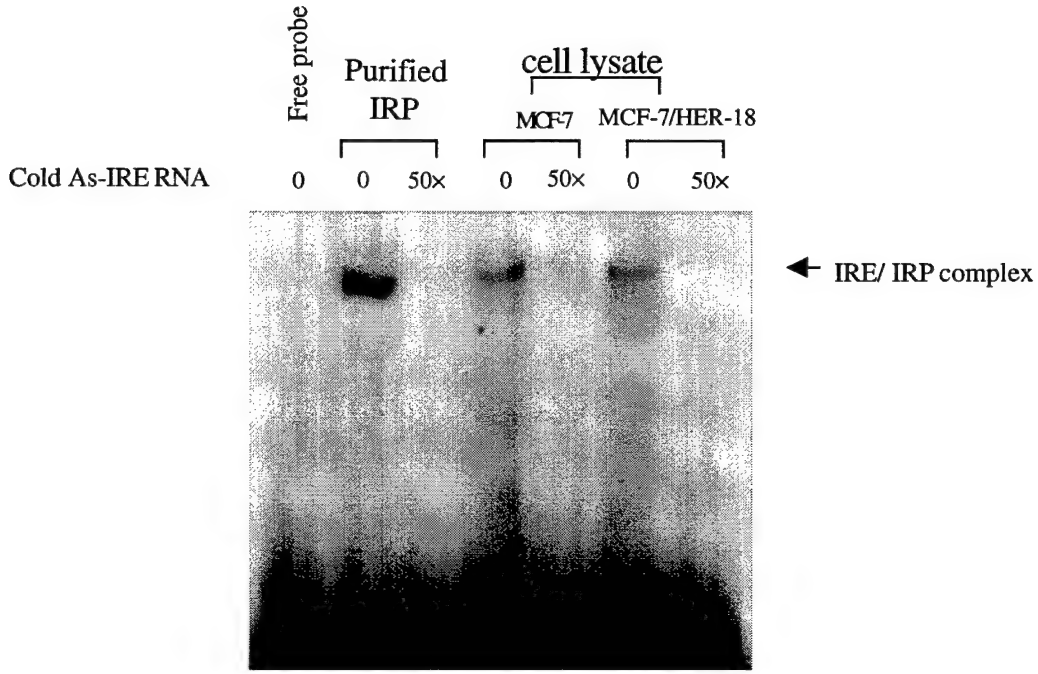


Fig.5 The result of RNA retardation assay using the radioactive As-IRE-2 probe.

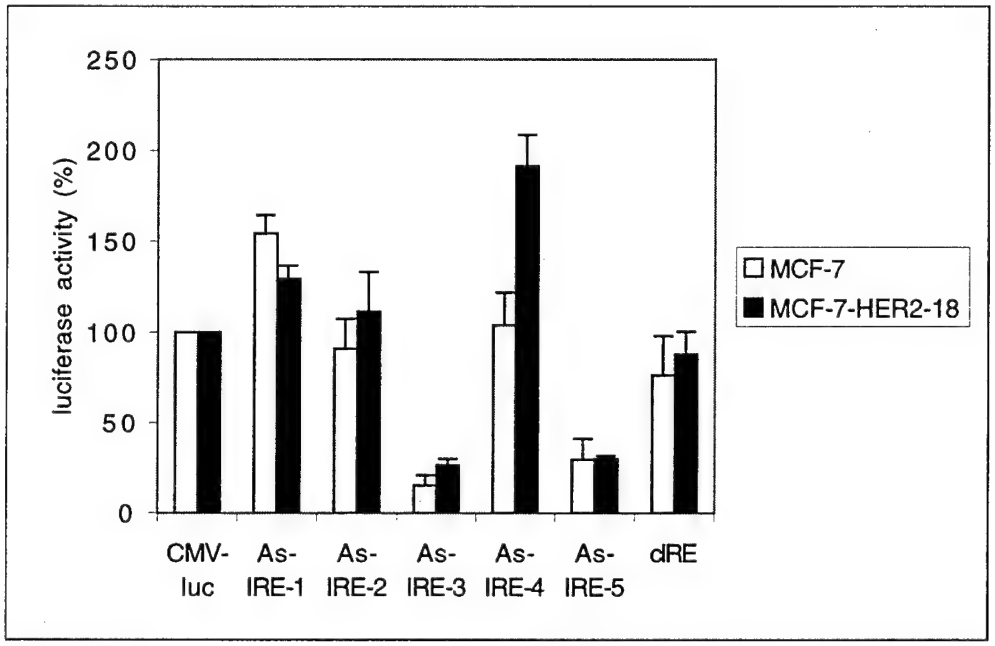


Fig.6A The result of luciferase assay after 48h transfection

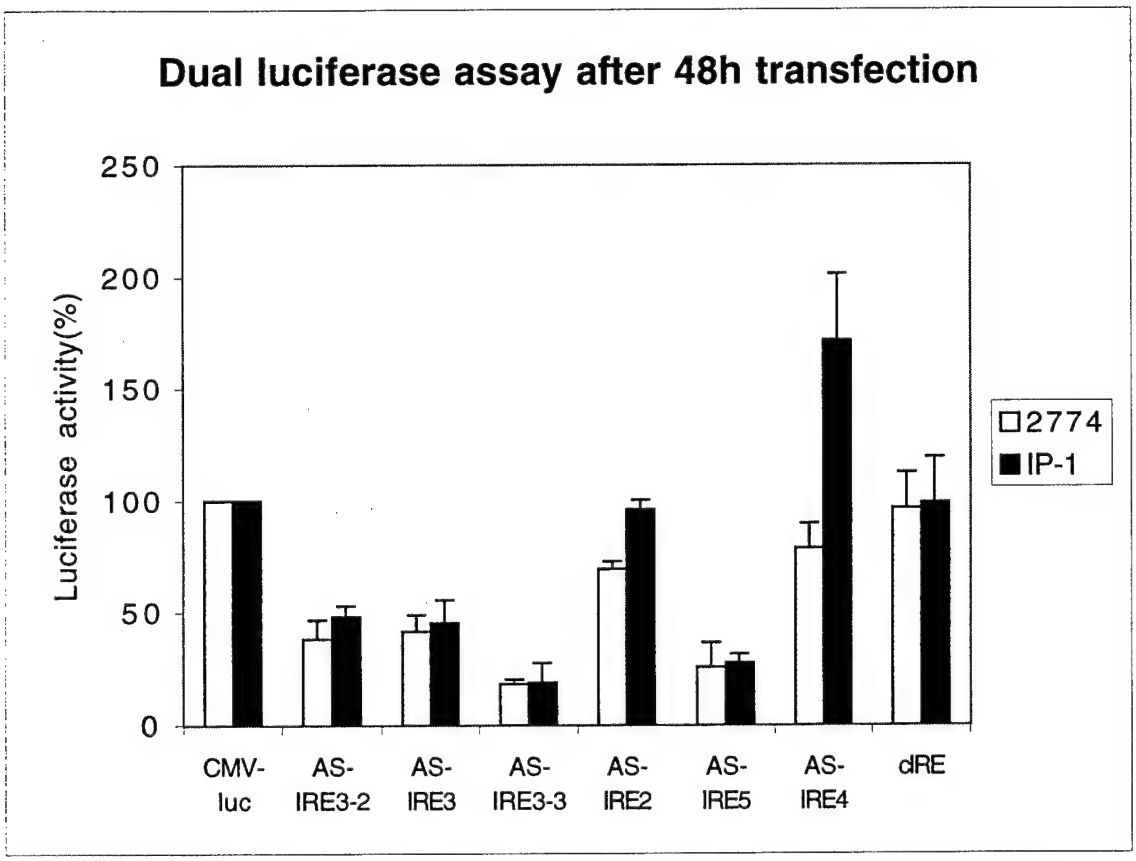
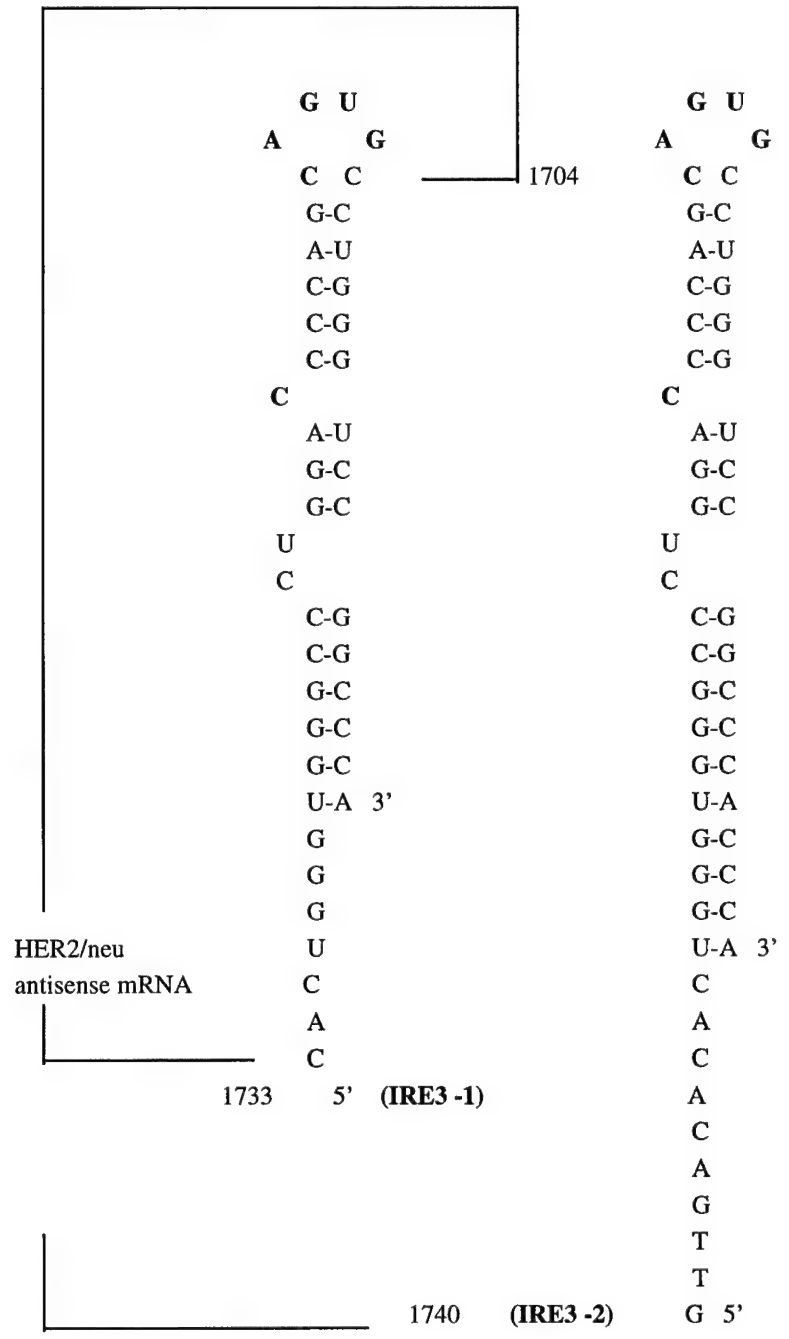
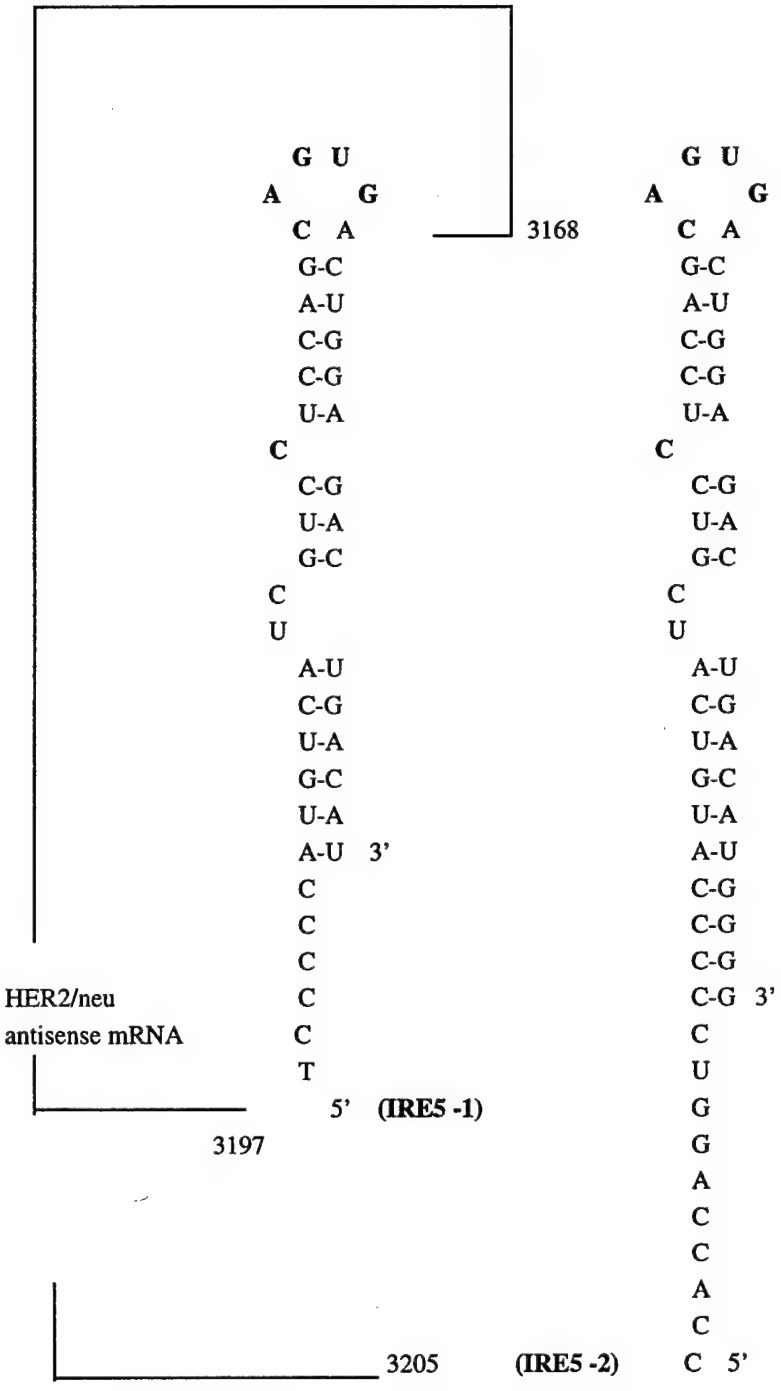


Fig. 6B The result of luciferase assay after 48h transfection. The firefly luciferase activity is normalized by renilla luciferase activity.



(**IRE3-3**: add a 41bases AS-HER2 tail based on IRE3-1)

Fig. 7A The structure of As-IRE3-1, As-IRE3-2 and As-IRE3-3.



(**IRE5-3**: add a 41bases AS-HER2 tail based on IRE5-1)

Fig. 7B The structure of As-IRE5-1, As-IRE5-2 and As-IRE5-3.

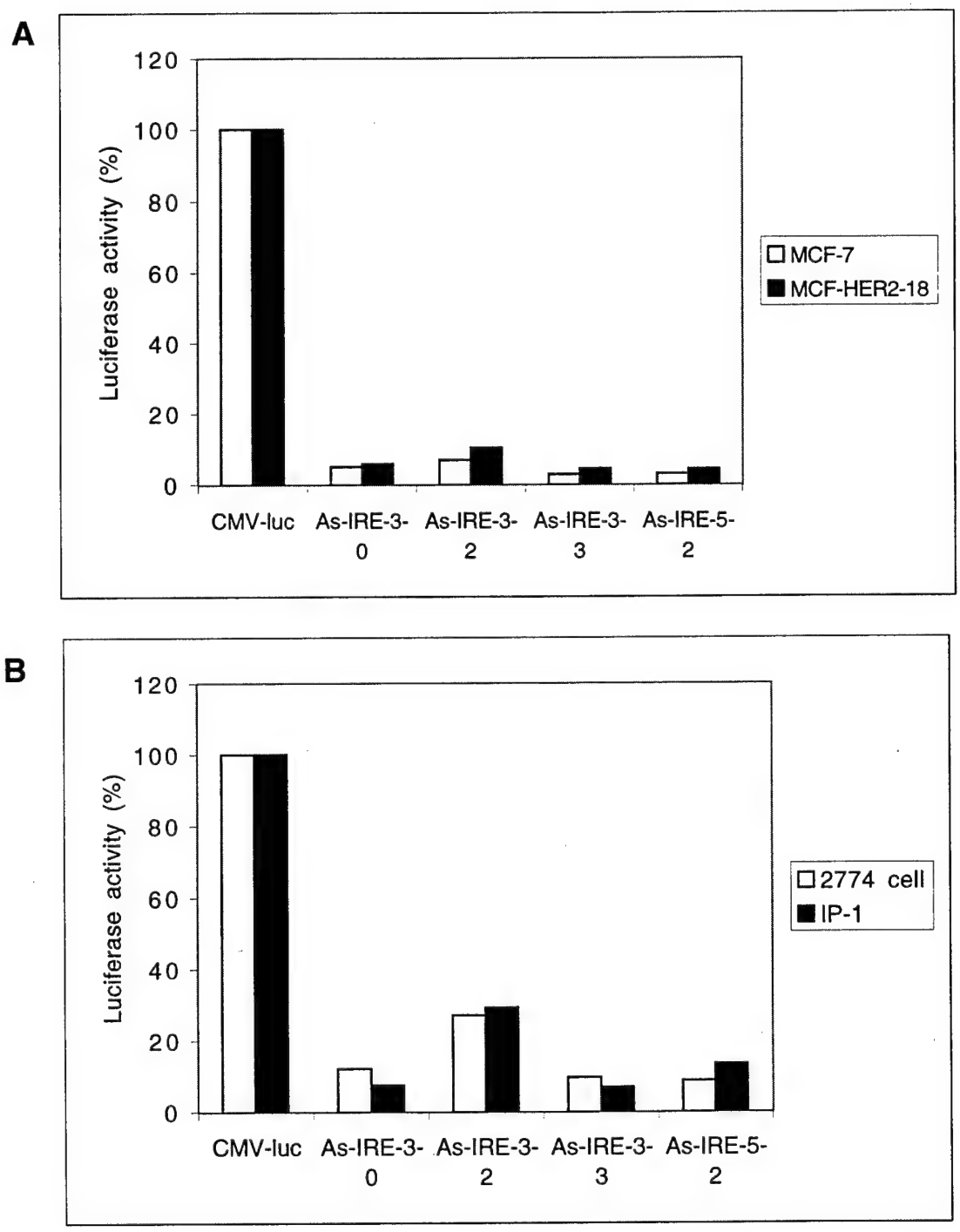


Fig. 8 The result of luciferase assay after 48h transfection.

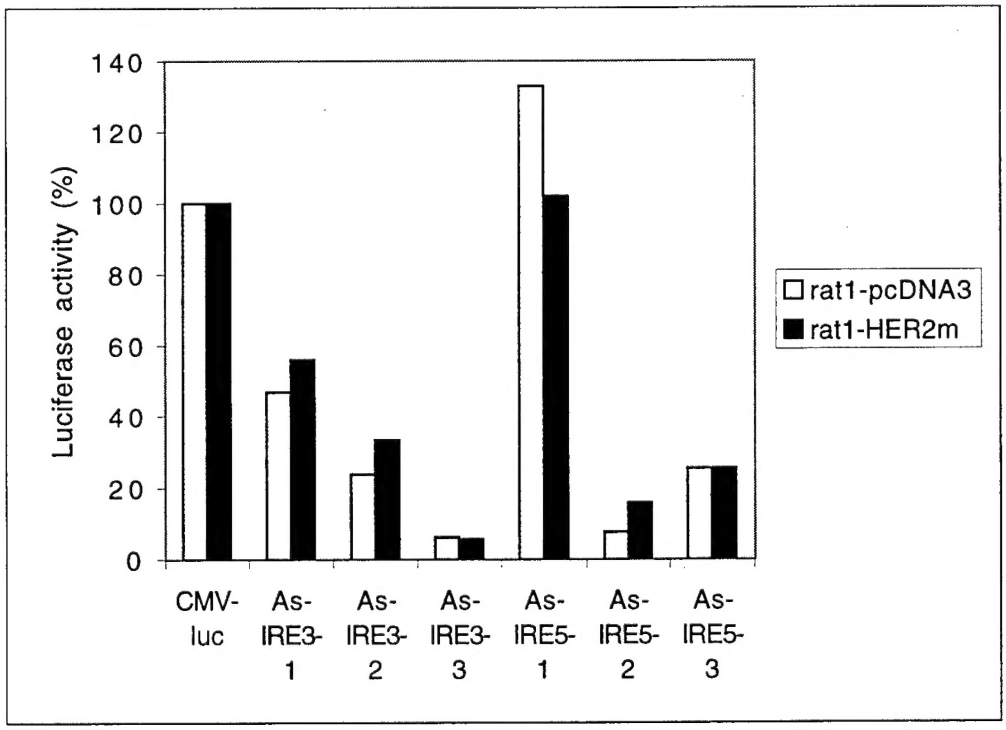


Fig. 9 The result of luciferase assay after 48h transfection.

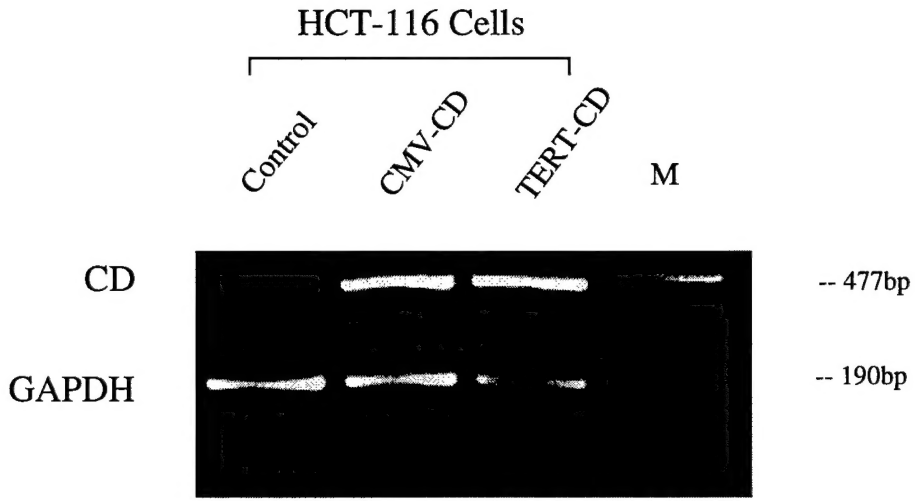


Fig.10 The result of RT-PCR after 48h Transfection

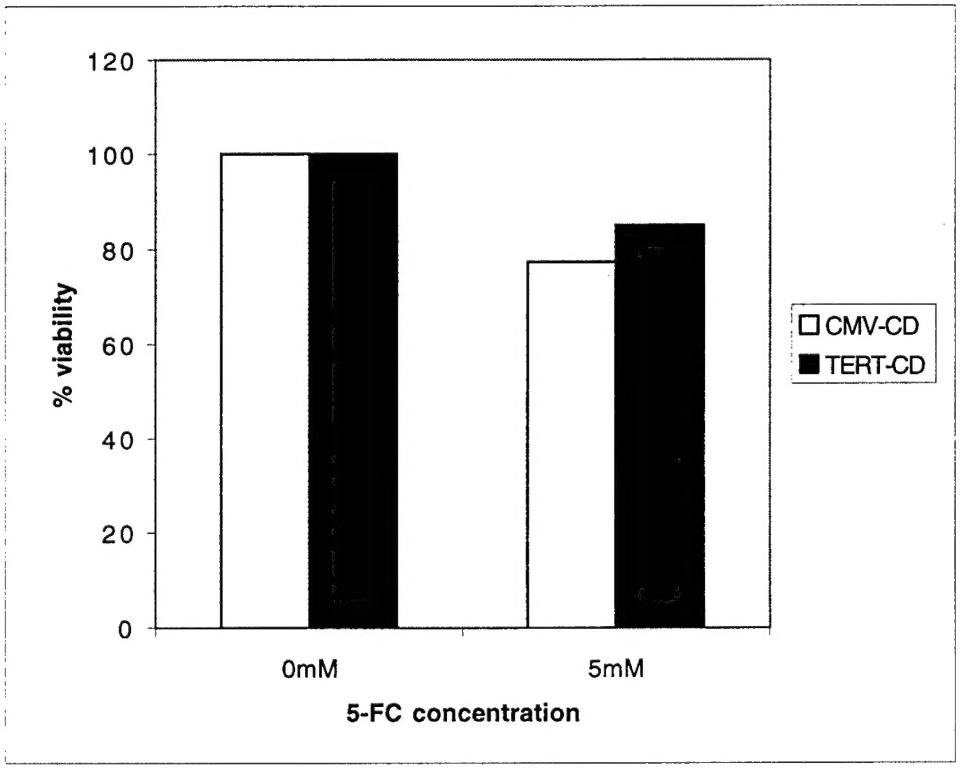


Fig.11A The result of MTT assay after 5-FC treatment for 48h in transfected HCT116 cells.

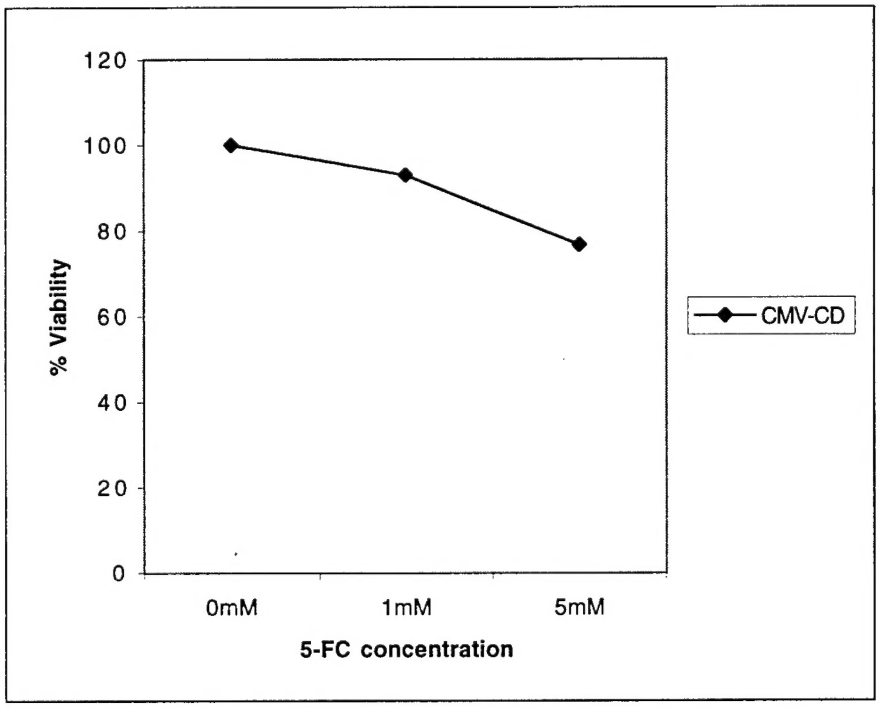


Fig.11B The result of MTT assay after 24h 5-FC treatment in MCF-7/CMV-CD stable cell line.